PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 4 : G01N 33/543	A1	(11) International Publication Number: WO 87/ 06708
		(43) International Publication Date: 5 November 1987 (05.11.87)

(21) International Application Number: PCT/US87/01018

(22) International Filing Date: 24 April 1987 (24.04.87)

(31) Priority Application Number: 856,942

(32) Priority Date: 28 April 1986 (28.04.86)

(33) Priority Country: US

(71) Applicant: BIO-METRIC SYSTEMS, INC. [US/US]; 9932 West 74th Street, Eden Prairie, MN 55344 (US).

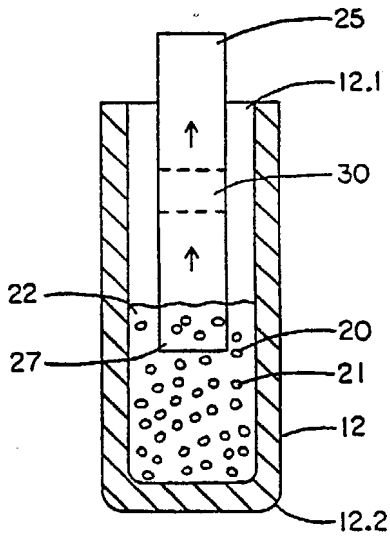
(72) Inventor: CHUDZIK, Stephen, J. ; 3151 Hays Street N.E., Minneapolis, MN 55418 (US).

(74) Agents: BAUMAN, Mary, P. et al.; Fredrikson & Byron, 1100 International Centre, 900 Second Avenue South, Minneapolis, MN 55402 (US).

(81) Designated States: AT (European patent), AU, BE (European patent), CH (European patent), DE (European patent), FR (European patent), GB (European patent), IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent).

Published
With international search report.

(54) Title: RAPID ASSAY INVOLVING SOLID PHASE RECEPTORS



(57) Abstract

Assay kits and methods which can be used to rapidly determine the presence or absence of minute quantities of analyte are shown. The kits and methods employ a predetermined quantity of a labeled ligand-receptor pair member, a first solid substrate (25) carrying a reaction zone (30) that contains a detection system responsive to the label, and a liquid-suspendable second solid substrate (20) having immobilized thereon a member of a ligand-receptor pair and having means for binding the labeled ligand-receptor pair member in relation to the quantity of analyte present in a sample. The label thus bound is substantially physically inhibited from reacting with the detection system carried by the first solid substrate (25).

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria
AU	Australia
BB	Barbados
BE	Belgium
BG	Bulgaria
BJ	Benin
BR	Brazil
CF	Central African Republic
CG	Congo
CH	Switzerland
CM	Cameroon
DE	Germany, Federal Republic of
DK	Denmark
FI	Finland
FR	France
GA	Gabon
GB	United Kingdom
HU	Hungary
IT	Italy
JP	Japan
KP	Democratic People's Republic of Korea
KR	Republic of Korea
LI	Liechtenstein
LK	Sri Lanka
LU	Luxembourg
MC	Monaco
MG	Madagascar
ML	Mali
MR	Mauritania
MW	Malawi
NL	Netherlands
NO	Norway
RO	Romania
SD	Sudan
SE	Sweden
SN	Senegal
SU	Soviet Union
TD	Chad
TG	Togo
US	United States of America

RAPID ASSAY INVOLVING SOLID PHASE RECEPTORS

BACKGROUND OF THE INVENTION

The present invention relates to an assay kit and method for determining the presence of microorganisms, chemicals, and other analytes in physiological and biological fluids.

The traditional methods of detecting and identifying microorganisms in physiological and biological fluids require cell culturing on laboratory media (sometimes followed by susceptibility testing to determine resistance to a particular antibiotic), identification of the organism by detection of serum antibodies against the organism, or isolation of the organism from infected tissues. These methods may be difficult to perform early enough to aid in patient management and diagnosis. In some cases the microorganism cannot be isolated or cultured. Development of rapid detection methods which are sensitive enough to determine if a particular microorganism is present in body fluid or biological fluids and which can be easily and rapidly performed in a clinic or doctor's office would greatly aid in the diagnosis of illnesses induced by such organisms.

Procedures for qualitatively determining the presence of chemical substances in solutions are common. Many of these procedures, however, are long and tedious, and are highly susceptible to human error. When the chemical substance whose presence is

suspected is related to a pathological process of a living organism or a particular drug, there is a need for a rapid and accurate assay so that early treatment can begin.

The present invention provides an assay kit and method capable of performing an assay to detect microorganisms or analytes in less than thirty minutes. No lab culturing of a microorganism is required, and a positive intense detectable signal is produced to alert the user to the presence of a particular microorganism in the physiological fluid being tested. Other drugs, chemicals and the like can be rapidly detected using the invention. No additional instrumentation is necessary for interpretation of results.

SUMMARY OF THE INVENTION

The present invention provides an assay kit and method for the detection of chemicals and other analytes that are members of ligand-receptor pairs. The invention employs a predetermined quantity of a labeled member of a ligand-receptor pair and a first solid substrate containing a reaction zone carrying a detection system responsive to said label to produce a detectable signal. The invention also employs a second solid substrate suspendable in a liquid and upon which is immobilized a member of a ligand-receptor pair, and means for binding the labeled ligand-receptor pair member to the second solid substrate in relation to the presence or quantity of analyte in the sample. The binding of the labeled ligand-receptor pair member substantially physically inhibits the label from molecularly interacting with the detection system contained in the reaction zone of the first solid substrate to produce a detectable signal..

In one embodiment of the invention, a predetermined quantity of a labeled member of a ligand-receptor pair is incubated with a sample suspected of containing an analyte that is also a member of the ligand-receptor pair in a liquid in which is suspended a solid substrate having immobilized thereon a member of the ligand-receptor pair. Another solid substrate, containing a reaction zone as above described is then contacted with the reaction mixture. The suspended solid substrate carries means for binding the labeled ligand-receptor pair member in relation to the quantity of analyte in the sample and thus, substantially physically inhibit the label from molecularly interacting with the detection system contained in the reaction zone.

In another embodiment of the invention, the suspendable, solid substrate having immobilized thereon a member of a ligand-receptor pair is uniformly suspended in a liquid and incubated with a labeled member of the ligand-receptor pair and sample suspected of containing analyte. Another solid substrate having a first end and carrying a reaction zone spaced from the first end of the substrate is contacted at its first end with the liquid-solid substrate admixture, and the liquid containing unbound labeled ligand-receptor pair member is allowed to flow upwardly through the reaction zone. The reaction zone contains means for binding and thereby concentrating the labeled ligand-receptor pair member and also includes a detection system responsive to said label. Unbound labeled ligand-receptor pair member carried by liquid flow into the reaction zone reacts with the signal detection system to produce a detectable signal.

In another embodiment, the invention comprises an assay kit for an analyte, the kit containing a liquid-suspendable, particulate, second solid substrate having immobilized thereon a member of a ligand-receptor pair, a predetermined quantity of a labeled member of the ligand-receptor pair, means for binding the labeled ligand-receptor pair member to the particulate substrate in relation to the quantity of analyte present in the sample, and a first solid substrate having a first end to be contacted with a liquid in which the second solid substrate is suspended to allow the liquid to flow upwardly therethrough, the first substrate having a reaction zone spaced from its first end, said reaction zone including means for binding and thereby concentrating the labeled ligand-receptor pair member thereon and having a detection system responsive to said label to produce a detectable signal.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic view, in cross-section, showing a step in the method of the invention;

Figure 2 is an enlarged, broken away view of a solid substrate shown also in Figure 1; and

Figure 3 is a schematic, cross-sectional view of a step in a method according to another embodiment of the invention.

DESCRIPTION OF PREFERRED EMBODIMENTS

As used herein, "ligand-receptor pair" refers to a pair of compounds of which one, a "receptor" is capable of recognizing a particular spacial and polar organization of the other ("ligand") or portion thereof, and is capable of binding to that compound. For

various ligands, illustrative receptors forming the other half of a ligand-receptor pair include antibodies, enzymes, lectins, Fab fragments, complementary nucleic acids and the like. Commonly, the receptor will be an antibody and the ligand will act as an antigen or hapten. Desirably, the analyte or an analyte derivative will be the ligand. As used herein, "analyte derivative" means a chemical derivative of an analyte that retains the capacity to bind to the other member of a ligand-receptor pair in competition with the analyte.

By "labeled member of a ligand-receptor pair" is meant a conjugate of a ligand-receptor pair member with a chemical label such as an enzyme, a fluorescent compound or other detectable chemical species, the conjugate retaining the capacity to bind to the other member of the ligand-receptor pair, and the enzyme or other detectable label continuing to have a capacity of being detected by a detector system (which may be a separate chemical reaction system) to provide a perceptible signal. "Detector," "label detector," "detection system," and the like, as exemplified below, refers to a chemical system that provides perceptible signals, commonly electromagnetic radiation or absorption of the same leading to perceptible fluorescense, color changes and the like, when contacted with a specific enzyme or other label.

The invention is useful in detecting a broad range of analytes. U.S. Patents 4,374,925 and 3,817,837 set out excellent lists of analytes which are part of specific binding pairs, the teachings of which patents are incorporated herein by reference. Other examples of binding pairs include nucleic acids and their complementary nucleic acids. Analytes of

particular interest include viruses, bacteria and fungi and macromolecules, specific products and assemblages thereof, drugs, toxins, and products of living organisms.

This invention may be used with several types of ligand-receptor reactions. The reaction may be a competitive one in which the labeled ligand-receptor pair member competes with the analyte for binding to a limited quantity of binding sites of another member of the ligand-receptor pair, commonly antibody to the analyte. The competitive reaction may be performed in several ways within the context of this invention. Antibody to the analyte may be immobilized on the liquid-suspendable particulate solid substrate and labeled ligand-receptor pair member (analyte) and the analyte then may compete for binding sites on the substrate. Alternatively, a predetermined quantity of analyte may be bound to the particulate substrate. The labeled ligand-receptor pair member may be a labeled antibody to the analyte. When sample suspected of containing analyte is incubated with the labeled antibody and the suspended solid substrate, free analyte in the sample will compete with the analyte on the substrate for binding to the labeled antibody. If desired, the free analyte in the sample may be pre-reacted with the labeled antibody and the resulting material then may be added to the suspended substrate.

Another example of a ligand-receptor reaction system that may be used with this invention is often referred to as a "sandwich" assay. This system requires one ligand-receptor pair member commonly the analyte, to have at least two binding sites. The

labeled ligand-receptor pair member and the ligand-receptor pair member immobilized on the liquid-suspendable particulate solid substrate both bind to the analyte at different sites. When analyte-containing sample, labeled antibody, and the particulate substrate are incubated together, the analyte will bind to the particulate substrate and the labeled ligand-receptor pair member will then bind to the analyte in relation to the amount of analyte in the sample. The bound label will thus be substantially physicially inhibited from reacting with the label detection system carried by the other solid substrate as described above.

A preferred embodiment of the invention is shown in Figure 1. A reaction vessel, typified as a transparent hollow column of glass or the like designated (12), has an open top end (12.1) and a closed bottom end (12.2). The reaction vessel contains a solid particulate substrate (20) such as particles of agarose, polyacrylamide, glass or cellulose fibers, substantially uniformly suspended in a liquid (22). The liquid is compatible with the ligand-receptor reaction and the detection system contained in the reaction zone, and to be used in a particular assay. The liquid is commonly phoshate buffered saline ("PBS") or the sample itself. If the sample is a liquid the reaction vessel may contain the suspendable solid substrate and the labeled lignd-receptor pair member in a dry form.

The solid substrate (20) is comprised of discrete small beads or particles (21) that have a high specific surface, that are capable of being suspended in the liquid and that have the ability to bind

antibody or other substances which constitute members of a ligand-receptor pair.

Shown in Figure 1 and in greater detail in Figure 2 is one embodiment of the invention that utilizes a first solid substrate (25) comprising an elongated strip having a first end (27) for contact with the ligand-solid substrate suspension (20) and having a reaction zone (30) spaced from the first end (27) a sufficient distance to avoid contact of the reaction zone with the suspension (20) when the first end (27) contacts the liquid surface (23). Although the particles of the solid substrate (20) may contact the first end (27), as shown in Figure 2, the lower end (27) of the strip acts as a filter to restrain upward movement of the particles (21).

The solid substrate (25) in this embodiment is bibulous in that it allows the liquid to be drawn upwardly through the reaction zone by capillary action. The solid substrate (25) may be made of filter paper or other fibrous, particulate or porous material that has the capacity to draw up liquid. In one embodiment, the substrate is a predetermined size so that a predetermined volume of liquid flows through the reaction zone. The reaction zone desirably has the ability to bind antibody or other substances that constitute a member of a ligand-receptor pair and it also contains components of the detection system reactive with the label to produce a detectable signal.

The reaction zone contains binding means for binding the labeled ligand-receptor pair member to thus concentrate the unbound labeled ligand-receptor pair member in the reaction zone. Such binding means may comprise an antibody or an antigen or any member

of a ligand-receptor pair to which the labeled ligand-receptor pair member will bind.

The signal-producing means carried by the reaction zone is responsive to the label on the ligand-receptor pair member to produce a detectable signal. The label desirably is an enzyme. Several convenient, known chromagens are available which produce visible color when added to their specific enzymes and substrates. For example, o-phenylenediamine and o-dianisidine have been used as peroxidase and glucose oxidase chromagen. Napthol-MX-phosphate is used as a chromagen for alkaline phosphatase. Yolken, R.N., "Solid-Phase Enzyme Immunoassays for the Detection of Microbial Antigens in Body Fluids," in Manual of Clinical Microbiology, pgs. 949-957 (1985) contains a more detailed description of these chromagens.

Another embodiment of the invention is shown in Figure 3. In this embodiment, the entire first solid substrate (28) can be contacted with the suspension. That substrate (28) includes a reaction zone (30) containing a detection system responsive to the label of the labeled ligand-receptor pair member. The components of the detection system are desirably substantially insoluble in the reaction mixture. "Substantially insoluble" in this context means that the components of the detection system either remain attached to the reaction zone via chemical bonds when the solid substrate carrying the reaction zone is contacted with the liquid suspension, or the component themselves are so sparingly soluble in the liquid as to substantially remain in place in the reaction zone when the latter is contacted with the liquid suspension.

Example 1 - Detection of Phenobarbital

A suspendable, solid substrate is produced comprising cross-linked agarose gel particules (60-140 microns, 4% agarose by weight in water) (Sepharose CL-4B, a trademarked product of Pharmacia Corp.) The "CL-4B" signifies 4% agarose by weight. IgG anti-phenobarbital is covalently coupled to the particles, as described below. The labeled ligand-receptor pair member used is horseradish peroxidase ("HRP") - polyethylene glycol ("PEG")-phenobarbital. The other solid substrate containing a reaction zone is comprised of a strip of Whatman #17 chromatography paper 1/4" by 2 3/4" containing, midway between its ends, a 1/4" square of the same paper to which IgG anti-phenobarbital and glucose oxidase ("G.O.") have been immobilized. (Procedure for preparation is described below.) The bottom 1/2 of the strip is impregnated with a 50 microgram per milliliter O-dianisidine solution and dried.

One-hundred microliters("uls") of a 1:1 suspension by volume of Sepharose CL-4B-IgG anti-phenobarbital particles in PBS buffer is placed in each of three test tubes. 100 uls of an 18% glucose solution, and 400 uls phosphate buffered saline ("PBS") containing 25 uls of a 0.50 mg/ml HRP-PEG-Phenobarbital solution is added to each test tube. To one tube 2.5 micrograms ("ugs") of sodium phenobarbital is added as a positive control. Sample suspected of containing phenobarbital is added to the second tube. The third tube is the negative control. The tubes are agitated and allowed to stand for two minutes, whereupon, the bottom ends of the strips are dipped into the resulting suspensions. Liquid from the suspensions is allowed to flow up the strips by

capillary migration, flowing through the reaction zones and continuing to the top of the strips. When the liquid reaches the tops of the strips (about four minutes), the strips are removed and examined for color development.

The reaction zone of the strip placed in the positive control suspension becomes dark brown, while the reaction zone of the strip placed in the other control solution remains white. Analyte present in the sample causes the reaction zone of the strip placed in the sample suspension to become brown.

The IgG preparation may be immobilized on the Sepharose CL-4B or Sepharose CL-6B ("CL-6B" signifies 6% agarose by weight in water) by the following method. Ten mls of Sepharose CL-4B or Sepharose CL-6B gel are transferred to a scintered glass funnel and washed successively with 50% dioxane, dioxane and dry dioxane. The gel is then packed into a large tube and reacted with 1 gram of carbonyldiimidazole dissolved in 20 mls of dry dioxane for 30 minutes at room temperature with agitation. The thus activated gel is then transferred back to the scintered glass funnel and washed successively with dioxane, 50% dioxane and cold 0.1M borate buffer, pH 9.0. The tube is rocked at 4°C for 24 hours. The gel is then washed extensively with PBS and stored in PBS at 4°C.

The HRP-PEG-Phenobarbital conjugate was prepared as follows. Additional amine groups were added to HRP by first oxidizing 2 mgs HRP in .05 ml of 1mM sodium acetate, pH 4.5, by the addition of 0.1 ml of a 0.1M sodium periodate solution. This reaction proceeded at room temperature for 30 minutes followed by a 24 hour dialysis against 1mM sodium acetate, pH 4.5.

After dialysis, 0.2 mls of a PEG diamine of approximately 600 molecular weight was added and the pH adjusted to 9.5. The reaction mixture was stirred for two hours at room temperature. The resulting Schiff base was reduced by the addition of 0.1 ml of a 4mg/ml sodium borohydride solution. The reaction mixture was allowed to stand for 6 hours at 4°C. The HRP-PEG (NH_2) was dialyzed extensively against PBS.

Six grams of the sodium salt of phenobarbital were dissolved in 72 mls of dry dimethylformamide ("DMF"). 5.52 grams of Ethyl-5-bromovalerate were added and the solution stirred at room temperature for 24 hours. 1.2 grams of potassium iodide were added and the reaction mixture stirred for 24 hours at room temperature. The resulting phenobarbital ethyl ester was purified on a silica gel column.

The ethyl ester was saponified to the free acid by dissolving in 30 mls Tetrahydrafuran, 15 mls 1N HC and 6 mls H_2O, stirring for 20 hours at room temperature and extracting the aqueous phase. The free acid was converted to the Sulfo-NHS (N-hydroxy sulfo succinimide) by a N,N'-Dicyclohexylcarbodiimide ("DCC") catalyzed reaction in DMF. The DMF is subsequently pulled off with a vacuum pump.

5 mgs HRP-PEG (NH_2) in 1 ml 0.1M borate buffer, pH 9.0, were added to 4 mgs of the Phenobarbital-Sulfo NHS compound and stirred for 45 minutes at room temperature. The conjugate was dialyzed extensively against PBS.

The IgG anti-Phenobarbital and G.O. squares were prepared as follows. 1/4" squares were cut from Whatman #17 chromatography paper. The squares were dried at 105°C for 45 minutes, cooled in a dessicator

and transferred to an appropriate vessel. The squares were activated with 1 gram of 1,1' carbonyldiimidazole dissolved in 35 mls dry dioxane per one hundred squares. The squares were activated for 45 minutes at room temperature with agitation. The squares were washed several times with cold d H_2O, and again several times with 0.1M borate buffer, pH 9.0. After washing, the squares were reacted with a solution consisting of 35 mls 0.1M borate buffer, pH 9.0, 20 mgs IgG and 2 mgs G.O. for every 100 squares. The reaction was allowed to proceed for 20 hours at 4°C with rocking. After the reaction was completed, the disks were washed several times with cold PBS and stored in PBS at 4°C.

Example 2 - Detection of T_2 Toxin

In this example, the suspendable, solid substrate is comprised of Sepharose CL-6B particles (particle size 45-165 microns) to which monoclonal antibodies against T_2 toxin have been covalently coupled, as described below. The labeled ligand-receptor pair member used is G.O. labeled T_2 toxin. The other solid substrate is comprised of a strip of Whatman #17 chromatography paper 1/4" by 4 1/4", divided into three contiguous segments, the middle segment comprising a reaction zone. The end segments are of equal dimension (1/4" x 2"), the lower segment being impregnated with a 50ug/ml 0-dianisidine solution and then dried. The reaction zone is a 1/4" square of the same paper to which T_2 antibodies and HRP have been covalently coupled, and to which an 18% glucose solution is dried.

Into each of three test tubes is added 25 uls Sepharose 6B-IgG anti-T_2 gel. 900 uls PBS, 50 uls methanol and 20 uls of a 0.10 mg/ml G.O.-T_2 solution

is then added to each vessel. To one tube 75 ngs of T_2 toxin is added as a positive control. Sample suspected of containing analyte is added to the second tube. The third tube is left untreated and acts as a negative control. The tubes are agitated and allowed to stand for 2 minutes, whereupon the bottom ends of the strips are dipped into the resulting suspensions. Liquid from the suspension is allowed to flow up the strips, through the reaction zones, to the top of the strips. When the liquid reaches the top (about five minutes), the strips are removed and examined for color development. The reaction zone of the positive control strips becomes brown, while the reaction zone of the negative control strip remains colorless. Analyte present in the sample strip causes the strip to become brown.

The T_2 antibody/HRP squares were prepared as follows. A stable HRP-PEG (NH_2) conjugated was prepared as described in Example 1. 1/4" squares of filter paper were cut and activated in the same manner as described in Example 1. After the squares had been activated and washed, they were reacted with a solution consisting of 35 mls 0.1M borate buffer, pH 9.0, 20 mgs IgG and 2 mgs HRP-PEG (NH_2) for every 100 squares. The reaction was allowed to proceed for 20 hours at 4°C with rocking. After the reaction was completed, the disks were washed extensively with cold PBS and stored in PBS at 4°C.

The G.O.-T_2 toxin conjugate can be prepared in the following manner. Twenty mgs of T_2 toxin are dissolved in 10 mls of pyridine. Five mgs succinic anhydride are added and the reaction is stirred at

room temperature for 24 hours. The pyridine solution is evaporated to dryness in vacuo. The resulting hemi-succinate is converted to the sulfo-NHS by a DCC catalyzed reaction in DMF. The DMF solution is evaporated to dryness in vacuo and the resulting T_2 - sulfo NHS compound is used for coupling to G.0.

Five mgs G.0. are dissolved in 1 ml 0.1M Carbonate buffer, pH 8.9. This solution is added to 2 mgs of the T_2 sulfo-NHS compound and the reaction mixture stirred for 45 minutes at room temperature. The G.0.-T_2 conjugate is dialyzed extensively against PBS.

Example 3 - Detection of Cholera Toxin

In this example, the suspendable, solid substrate is comprised of Sepharose CL-4B particles to which choleragenoid antibodies have been covalently immobilized, as described below. The labeled ligand-receptor pair member used is a HRP labeled choleragenoid. The other solid substrate is comprised of a strip of Whatman #17 chromatography paper 1/4" by 4 1/4", divided into three contiguous segments, the middle segment of which constitutes the reaction zone. The end segments are of equal dimension (1/4" x 2"), the lower segment being impregnated with a 50 ug/ml 0-dianisidine solution and dried. The reaction zone comprises a 1/4" square of the same paper to which choleragenoid antibodies and G.0. have been covalently coupled.

Into each of three test tubes is added 100 uls Sepharose 4B-IgG anti-choleragenoid gel. 800 uls PBS, 100 uls of an 18% glucose solution and 15 uls of a 0.10 mg/ml HRP-choleragenoid are added to each tube. To one tube 1 ug Cholera toxin beta subunit is added as a positive control. Sample suspected of containing

analyte is added to the second tube. The third tube is untreated and is the negative control. The reaction tubes are agitated and allowed to stand for 2 minutes, whereupon the bottem ends of the strips are dipped into the resulting suspension. Liquid from the suspension flows up the strips, passing through the reaction zones. When the liquid reaches the tops of the strips, the strips are removed and examined for color development. The reaction zone of the positive control strip becomes brown, while the reaction zone of the negative control strip remains white. Analyte present in the sample causes the sample strip to become brown.

The HRP-Choleragenoid conjugate was prepared as follows. The carbohydrate portion of HRP is oxidized by sodium periodate, as described in Example 1. Cholera toxin beta subunit was purchased and repurified by chromatographing on a G-75 column. To 2 mgs of the oxidized HRP is added 2.9 mgs of the purified cholera toxin beta sub-unit. The pH is adjusted to 9.5 by the addition of sodium carbonate. After two hours of stirring at room temperature, the resulting Schiff base is reduced by the addition of 0.1 ml of a 4 mg/ml sodium borohydride solution. The reaction mixture is left to stand for 6 hour at 4°C. The crude conjugate is chromatographed on a G-75 column with fraction absorbances monitored at A_{280} and A_{403}.

Example 4 - Detection of Chlamydial Agent

In this example, the suspendable, solid substrate is comprised of Controlled Pore Glass-N-hydroxy succinimide (CPG-NHS) glycophase glass beads (125-177 uM particle size) to which monoclonal antibodies against Chlamydia have been covalently coupled, as described below. The labeled ligand-receptor pair

member is HRP-labeled Chlamydia. The other solid substrate is comprised of a strip of Whatman #17 chromatography paper, 0.5 cm x 5 cm, divided into three contiguous segments, the middle segment of which is comprised by a reaction zone. The end segments are of equal dimension, the lower segment being impregnated with a 50 ug/ml O-dianisidine solution and dried. The reaction zone is comprised of a 0.5 cm x 1 cm segment of the same paper to which G.O. and Chlamydia antibodies have been covalently coupled.

Into each of three test tubes is added 40 uls CPG-IgG anti-Chlamydia beads. 350 uls PBS, 100 uls of an 18% glucose solution and 10 uls of an 0.040 mg/ml HRP-chlamydia solution is added to each tube. To one tube 1 x 10^9 Chlamydial organisms are added to act as a positive control. Sample suspected of containing analyte is added to the second tube. Nothing else is added to the third tube and it provides a negative control. The reaction tubes are agitated and left to stand for two minutes, whereupon the bottem ends of the strips are dipped into the resulting suspensions. Liquid from the suspension flows up the strips, passing through the reaction zones. When the liquid reaches the tops of the strips (about 4 minutes), the strips are removed and examined for color development. The reaction zone of the positive control strip becomes brown, the reaction zone of the negative control strip remains white. Analyte present in the sample causes the sample strip to become brown.

The Chlamydial antigen was prepared as follows. 12 mls of a frozen chlamydia sample were placed in a flask along with 12 mls of PBS. The Chlamydia sample was heat-killed at 50°C with gentle agitation for 2 hours. The sample was sonicated using a Braunsonic

1510 sonicator, with 10, 200 watt bursts of 15 seconds, with a 30 second cool down between bursts. The sample was centrifuged for 15 minutes at 15,000RPM. 10 mls of supernatant were collected containing 1.5 mg protein as determined by a Bio-Rad protein assay using a BSA standard. This protein from the supernatant was used as the Chlamydial antigen.

The HRP-Chlamydia conjugate was prepared as follows. The carbohydrate portion of HRP was oxidized by sodium periodate as described in Example 1. 1.5 mgs of the Chlamydia antigen preparation were adjusted to pH 9.0 by the addition of 0.3M sodium carbonate. This solution was added to 3 mg of the oxidized HRP and the reaction mixture was stirred at room temperature for 2 hours. The Schiff base was reduced by the addition of 0.2 ml of a 4 mg/ml sodium borohydride solution. The reaction mixture was left to stand for 6 hours at 4°C.

The crude conjugate was dialyzed against PBS and then concentrated to 2.5 mls by ultra-centrifugation. The conjugate was chromatographed on a G-150 column with fraction absorbances monitored at A_{280} and A_{403}.

The IgG was immobilized on the solid substrate by the following method. An IgG preparation was dialyzed against 0.1M borate buffer, pH 9.0, for 24 hours at 4°C. After dialysis, the IgG was added to 0.4 gram CPG-NHS (5A pore diameter, 125-177 uM particle size). Two mls 0.1M Borate buffer, pH 9.0 was added and the reaction mixture rotated at 4°C for 36 hours. When the reaction was completed, the beads were washed extensively with cold PBS and stored in PBS at 4°C.

For many of the applications of this invention, the segment of the strip which contacts the reaction solution is impregnated with an O-dianisidine solution. The strips are prepared as follows. Whatman #17 paper is cut to the appropriate size and immersed in a solution consisting of 50 ug/ml O-dianisidine and 1% PEG-4000 in PBS. The strips are removed and air dried at room temperature in the dark.

Example 5 - Semi-quantitative Detection of Phenobarbital

In this example, the suspendable solid substrate is comprised of Sepharose CL-4B particles to which phenobarbital antibodies had been covalently immobilized, as described in Example 1. The labeled ligand-receptor pair member is an HRP-PEG-Phenobarbital conjugate. The other solid substrate is an individual reaction disk comprised of 1/4" diameter #17 Whatman chromatography paper which contains covalently immobilized IgG anti-Phenobarbital and glucose oxidase and has been impregnated with a 100 ug/ml O-dianisidine solution and dried.

Into each of four test tubes is added 25 uls Sepharose 4B-IgG anti-phenobarbital gel. 350 uls PBS, 100 uls of an 18% glucose soution and 30 uls of a 0.010 mg/ml HRP-PEG-phenobarbital solution is added to each tube. 0.5 ug Phenobarbital is also added to the second tube. To the third tube is added 1.0 ug phenobarbital. 2.5 ugs phenobarbital are added to the fourth tube. The reaction tubes are agitated and left to stand for 2 minutes, whereupon reaction disks are added to the reaction solutions. The reaction tubes are reagitated and allowed to stand for an additional three minutes. After this three minute incubation, the reaction disks are removed and examined for color

development. The reaction disk from the first tube remains white. The reaction disk from the second tube becomes light brown. The reaction disk from the third tube becomes a darker brown and the reaction disk from the fourth tube exhibits the darkest color.

While a preferred embodiment of the present invention has been described, it should be understood that various changes, adaptations and modifications may be made therein without departing from the spirit of the invention and the scope of the appended claims.

WHAT IS CLAIMED IS

1. A method of determining the presence, in a sample, of an analyte which is one member of a ligand-receptor pair, the method employing

a. a predetermined quantity of a labeled ligand-receptor pair member;

b. a first solid substrate having a reaction zone that contains a label detection system responsive to the label of the labeled ligand-receptor pair member to produce a detectable signal;

c. a liquid-suspendable second solid substrate upon which a member of the ligand-receptor pair is immobilized and having means for binding the labeled ligand-receptor pair member thereto in relation to the quantity of analyte in the sample, the label of the ligand-receptor pair member so bound being substantially physicially inhibited from molecularly interacting with the reaction zone of the first solid substrate; said method comprising:

i. incubating the sample and the labeled ligand-receptor pair member in a liquid suspension of the second solid substrate;

ii. contacting the first solid substrate with the liquid;

iii. allowing the unbound labeled ligand-receptor pair member to molecularly interact with the detection system contained in the first solid substrate; and

iv. perceiving the detectable signal if analyte is present in the sample.

2. The method of claim 1 in which the labeled ligand-receptor pair member is labeled analyte.

3. The method of claim 1 in which the ligand-receptor pair member immobilized on the second solid substrate is analyte.

4. The method of Claim 1 in which the reaction zone of the first solid substrate further contains means for binding the labeled ligand-receptor pair member.

5. The method of Claim 2 in which the first solid substrate comprises a bibulous material.

6. The method of Claim 3 in which the first substrate comprises an elongated strip having first and second ends, the reaction zone being spaced from each end.

7. The method of Claim 6 in which the first solid substrate is a predetermined size to convey, through the reaction zone, a predetermined volume of liquid.

8. The method of Claim 7 further comprising contacting the first end of the first solid substrate with the liquid and allowing the liquid to flow upwardly through the reaction zone by capillary action.

9. An assay kit to be used in determining the presence of an analyte that is a member of a ligand-receptor pair in a sample, comprising:

a. a predetermined quantity of a labeled ligand-receptor pair member;

b. a first solid substrate having a reaction zone that contains a label detection

system responsive to the label of the labeled ligand-receptor pair member to produce a detectable signal;

c. a liquid-suspendable second solid substrate upon which a member of the ligand-receptor pair is immobilized and having means for binding the labeled ligand-receptor pair member thereto in relation to the quantity of analyte in the sample, the label of the thus bound ligand-receptor pair member being substantially physicially inhibited from molecularly interacting with the detection system contained in the first solid substrate.

10. The assay kit of claim 9 in which the labeled ligand-receptor pair member is labeled analyte.

11. The assay kit of claim 9 in which the ligand-receptor pair member immobilized on the liquid-suspendable solid substrate is analyte.

12. The kit of Claim 9 in which the ligand-receptor pair member immobilized on the suspendable solid substrate is antibody to the analyte.

13. The kit of Claim 9 wherein the first solid substrate comprises a strip of bibulous material having the reaction zone containing the detection system spaced from its ends.

14. A method of determining the presence, in a sample, of an analyte that is one member of a ligand-receptor pair, the method employing

a. a liquid;

b. a predetermined quantity of a labeled ligand-receptor pair member, the label being part of a signal-producing system;

c. a solid substrate suspended in the liquid and upon which a member of a

ligand-receptor pair has been immobilized and having means for binding the labeled ligand-receptor pair member thereto in relation to the quantity of analyte in the sample;

d. an elongated second solid substrate having a first end adapted to be contacted with the liquid to allow liquid to flow upwardly therewithin, the substrate having a reaction zone spaced from its first end, said reaction zone including means for binding and concentrating the labeled ligand-receptor pair member and carrying a label detection system responsive to said label to produce a detectable signal; said method comprising:

i. incubating the liquid suspension with the sample and labeled ligand-receptor pair member;

ii. contacting the first end of the bibulous element with the liquid;

iii. allowing the liquid containing the unbound labeled ligand-receptor pair member to flow upwardly through the bibulous element into the reaction zone by capillary action, the detection system in the latter producing a detectable signal in the presence of the labeled ligand-receptor pair member.

15. The method of claim 13 in which the labeled ligand-receptor pair member is labeled analyte.

16. The method of claim 13 in which the ligand-receptor pair member immobilized on the liquid-suspendable solid substrate is analyte.

17. The method of claim 13 in which the means for binding the labeled ligand-receptor pair member is antibody to the label.

18. An assay kit to be used in determining the presence of an analyte, in a sample, that is a member of a ligand-receptor pair comprising:

a. a predetermined quantity of a labeled ligand-receptor pair member;

b. a liquid-suspendable solid substrate having immobilized thereon a first ligand-receptor pair member and having means for binding the labeled ligand-receptor pair member to the solid substrate in relation to the quantity of analyte present in the sample;

c. an elongated bibulous element having a first end adapted to contact a reaction mixture comprising the sample, labeled ligand-receptor pair member, the solid substrate and a liquid medium in which the solid substrate is substantially uniformly suspended to allow the liquid to flow upwardly into the bibulous element including; and

d. a reaction zone on the bibulous element spaced from the first end and having means for binding thereto the labeled ligand-receptor pair member, the zone including a label detection system responsive to said label to produce a detectable signal when the liquid flows there through by capillary action.

19. The kit of claim 17 in which the ligand-receptor pair member immobilized on the liquid-suspendable solid substrate is analyte.

20. The kit of claim 17 in which the labeled ligand-receptor pair member is labeled analyte.

21. The kit of claim 17 in which the means for binding and concentrating the labeled ligand-receptor pair member is antibody to the label.

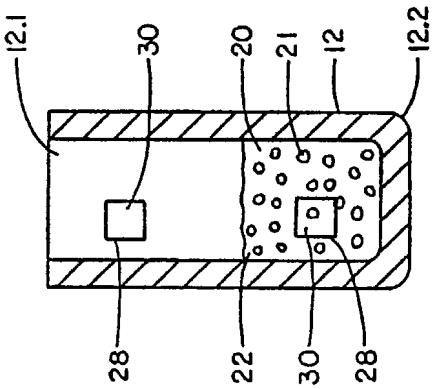


FIGURE 3

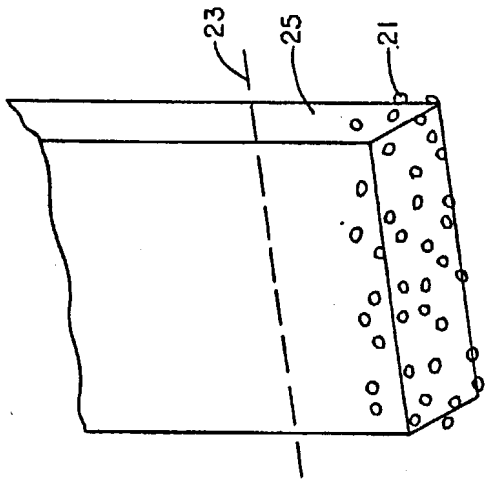


FIGURE 2

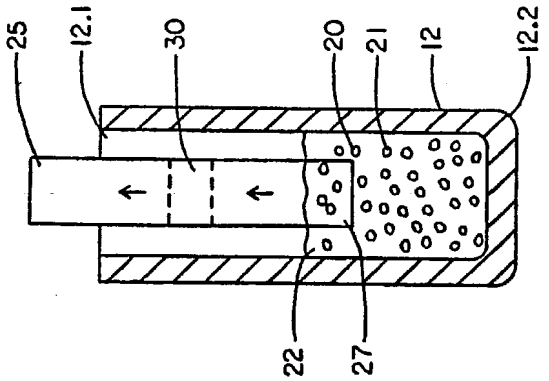


FIGURE 1

INTERNATIONAL SEARCH REPORT

International Application No PCT/US87/01018

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): G01N 33/543

U.S. CL: 422/56; 435/7,805; 436/501,510,533,534,810

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	422/56 435/7,805, 810 436/501, 518, 533, 534, 810

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
Y	US,A, 3,654,090 (SCHUURS ET AL) 04 April 1972. See column 3, lines 3-15	1-8,11, 14-17 & 19-21
Y	US,A, 4,094,647 (DEUTSCH ET AL) 13 June 1978. See abstract; column 3, lines 55-58 and 68; column 4, lines 1-3 and 35-44.	1-8 & 13-21
A	US,A, 4,447,526 (RUPCHOCK ET AL) 8 May 1984. See entire document.	
X,P	US,A, 4,587,102 (NAGATOMO ET AL) 6 May 1986. See column 8, line 62; column 10, line 46-column 11, line 12; column 16, lines 20-63.	9,10 & 12
A,P	US,A, 4,594,327 (ZUK) 10 June 1986. See entire document.	

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
27 July 1987	04 AUG 1987
International Searching Authority [1]	**Signature of Authorized Officer [20]**
ISA/US	David A. Saunders David A. Saunders